Lipid Biotechnology

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Supercritical Fluid Technology for Lipid Extraction, Fractionation, and Reactions

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1 INTRODUCTION

Critical fluids are substances held above their critical temperature (T_c) and pressure (P_c) or liquids sustained in their liquid state by the application of pressure, which can be used for the extraction of natural products or as an alternative reaction medium. By far the most utilized critical fluid has been supercritical carbon dioxide (SC-CO_2) or its liquified analogue (LCO_2), due to its benign effect on the environment, low toxicity, nonflammability, and compatibility with processed foodstuffs. Several well-known applications of the technology exist, including the decaffeination of coffee [1], extraction of hop essence for flavoring [2], production of spice and aroma concentrates [3], and isolation of natural antioxidants [4]. More recently, critical fluids have been applied for the production of fine particles, or as a versatile reaction medium, and for the modification of novel materials such as polymers and cements.

This chapter is concerned with applying critical fluids for the extraction, fractionation, and reaction of lipid moieties, particularly those operations which have implications for lipid biotechnology. An excellent example of combining these two technologies is the use of critical fluids with enzymatic catalysis to produce unique lipid materials. This will

Names are necessary to report factually on available data; however the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the products to the exclusion of others that may also be suitable.

be illustrated with examples from the author's research [5] and further discussed in Chapter 35. Both critical fluid technology and lipid biotechnology share in common the goal to modify natural products, thereby producing either a superior product or a process that is both consumer and environmentally acceptable. Today, it is estimated that there are over 44 production plants employing critical fluid technology throughout the world, and the technology is currently receiving additional interest with the development of the nutraceutical and functional food markets [6].

Supercritical fluid extraction (SFE) became an industrial reality in the early 1970s, but as we shall see later, it is a technique which has limitations with respect to the resolution that it can achieve between molecular species. Modern trends in supercritical fluid processing emphasize fractionation schemes that can separate lipid species having similar physical properties or the enrichment of target lipid compounds from complex oleochemical mixtures. Reaction chemistry in supercritical agents such as SC-CO_2 is not limited to enzymatically catalyzed transformations, but includes hydrogenation and hydrolysis of lipids utilizing mixtures of binary fluids [6] and subcritical water [7], respectively. Such processes support the hypothesis of "green" processing and products which are recognized by the general public as highly desirable.

In the next section, some of the fundamental principles which govern the application of critical fluids in SFE and supercritical fluid fractionation (SFF) will be discussed. The use of SC-CO_2 will be emphasized because of its use in industry, low cost, and the favorable critical properties. Then, the mechanics of SFE, generic equipment requirements, and examples of this technique will be presented. This will be followed by fractionation methodology utilizing columnar fractionation approaches and preparative chromatography. Finally, the use of the critical fluids as a reaction medium will be presented with examples of how this can be coupled with SFE and SFF.

It should be noted that critical fluids have also found widespread application for the analysis of lipids both in extraction (SFE) [8] and chromatographic mode (SFC) [9]. To cover this subject in detail would require a separate chapter; hence, we will only note this application as it supports research and development in the above-mentioned areas. Indeed, analytical-scale SFE and SFC can be used to model extraction and reaction processes in critical fluids and can be used in a combinatorial fashion [10] to improve extraction efficiency and select catalysts for use in supercritical media. However, their most important application in analytical chemistry has been as an alternative extraction technique to replace organic-solvent-intensive methodology, thereby reducing the use of hazardous solvents in the laboratory environment [11].

2 FUNDAMENTALS OF CRITICAL FLUID TECHNOLOGY AS APPLIED TO LIPIDS

The conditions defining a supercritical fluid (SF) are frequently described by a region of the phase diagram of pressure versus temperature for the common critical fluid, CO_2. This is shown in Figure 1, in which the SF region in the upper right-hand sector is defined by carbon dioxide's T_c (31°C) and P_c (1070 psi or 73 atm). It should be emphasized that this is an arbitrary definition, because others define the SF state as simply being above the critical temperature of a substance. This broader definition of a SF fluid acknowledges the fact that critical fluids such as carbon dioxide under modest compression can also interact with more volatile lipid species as well as higher-molecular-weight compounds (e.g., triglycerides).

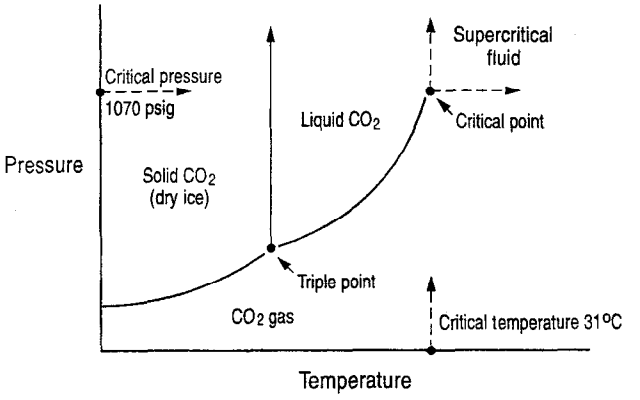

Figure 1. Phase diagram for carbon dioxide.

Movement across the arbitrary boundaries defined in Figure 1 can be quite easily affected by changing the temperature and pressure of the fluid. Thus, LCO_2 has also been used to extract natural products [12] but suffers from reduced extraction selectivity because its density can only be varied slightly as a function of temperature and pressure. On the other hand, SC-CO_2's density can be varied from that associated with a dilute gas (10^{-3} g/cm^3) to densities in excess of unity simply by altering the applied mechanical pressure. This "tunable" solvent power is one of the major attractive features in using supercritical fluids as processing agents.

Other factors besides CO_2's low cost, environmental compatibility, and low toxicity make it the most popular SF. The extraction fluxes obtained using SC-CO_2 are both a function of the solubility and diffusivity of the dissolved solutes (i.e., lipids) in CO_2; therefore, the mass transfer properties of SFs such as diffusivity and viscosity also play an important role in processes using SFs. For example, SF solvents exhibit self-diffusivities of the order of 10^{-3} cm^2/sec, whereas liquids have diffusion coefficients of approximately 10^{-6} cm^2/sec. This "gaslike" nature of SFs gives them superior penetration properties into substrates, such as oilseeds, relative to that obtained by using liquid solvents. Likewise, the viscosity of SF solvents are of the order of 10^{-4} g/cm · sec, two decades lower than those exhibited by liquids [13].

The mechanically adjustable solvent power of an SF can be nicely correlated with the aid of the solubility parameter concept, where the solubility parameter, δ, as defined by Giddings et al. [14] is given by

$$\delta = 1.25 P_c^{1/2} \frac{\rho_{r,f}}{\rho_{r,l}} \tag{1}$$

where $\rho_{r,f}$ is the reduced density of the fluid at actual experimental conditions, and $\rho_{r,l}$ is the reduced density of the fluid at infinite compression [i.e., the liquified gas (values range between 2.6 and 3.1)]. These reduced parameters are simply the fluid's actual density over the critical density of the fluid (~ 0.44 g/cm^3 for CO_2). A plot of the δ of several common gases as a function of pressure is illustrated in Figure 2. Here, it can be seen that as one compresses CO_2, its δ increases substantially as a function of pressure relative to the δ of nitrogen and helium at temperatures between 50°C and 70°C. Note that between 8000 and 10,000 psi, the δ of SC-CO_2 reaches values which match those exhibited by most

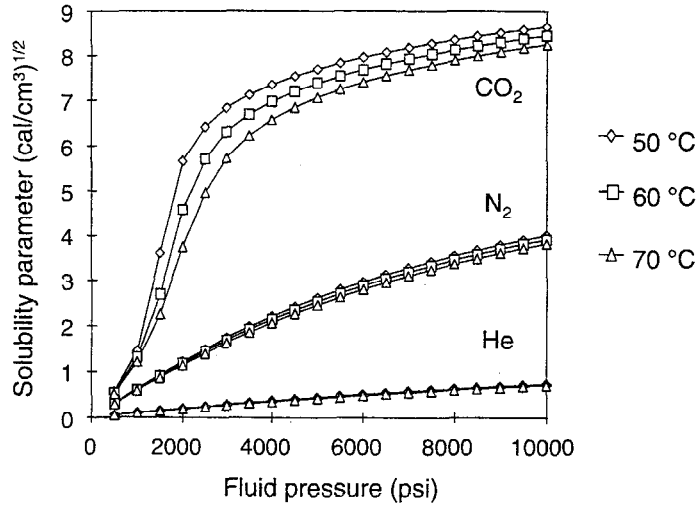

Figure 2. Solubility parameters of various supercritical fluids versus applied pressure.

lipid species [15]. It is under these conditions that the solubility of most lipid species is maximized in SC-CO_2 (i.e., $\delta_{r,f} = \delta_{\text{lipid solute}}$). This does not imply, however, that smaller but finite solubility levels are not attained in SC-CO_2 at lower levels of fluid compression.

The asymptotical values for CO_2's solubility parameter in Figure 2 correspond to reduced densities for CO_2 between 2.0 and 2.2. Table 1 lists solubility parameters associated with lipid-type species found in vegetable oil mixtures as well as the $\rho_{r,f}$ associated with extraction conditions to maximize their solubility in SC-CO_2. Note that the reduced densities for each solute (lipid) class is in the range of the asymptotic values for the solubility parameter for SC-CO_2. It is for this reason that SFE of oils at these high levels of compression yield extraction products (oils) that are equivalent to those obtained with liquid hydrocarbon solvents (i.e., hexane). Table 1 also illustrates a limitation of SFE.

Table 1 Solubility Parameters for Lipid Groups Found in Seed Oils and the Reduced Densities Required to Optimize Extraction of These Lipid Groups

Lipid type	Solubility parameter ($cal^{1/2}/cm^{3/2}$)	Reduced density
Hydrocarbons	8.34	2.08
Carotenoids	8.72	2.17
Tocopherols	8.86	2.21
Triglycerides	8.91	2.22
Ubiquinones	9.08	2.26
Fatty acids	9.10	2.27
Diglycerides	9.45	2.35
Sterols	9.52	2.37
Monoglycerides	10.2	2.54

Note that the reduced density interval between the lipid classes is relative small (only 0.44), suggesting that fractionation of individual lipid species can be difficult at best. This is in agreement with the observation that a difference in δ values of at least 2.5 is required for complete separation of one species from another.

The theoretical considerations presented above translate into significant differences in lipid solubility in SC-CO_2 as a function of pressure and temperature. As shown in Figure 3, extraction of triglycerides from soybean oil [16] reaches levels of only 3 wt% at 40°C over a pressure range from 100 to 1000 bar. However, as the extraction temperature is increased, the reinforcing effect of solute (lipid) vapor pressure along with the solubility of the triglycerides in SC-CO_2 can yield a substantial increase in the yield of lipid species using extraction temperatures of 70–80°C. This increase in a solute's solubility in SC-CO_2 as temperature is increased as a function of pressure is an example of the well-known crossover phenomenon which occurs for many solutes in SFE [17].

The above-stated conditions are close to the recorded solubility maximum observed for lipid-type seed oil in SC-CO_2. Figure 4 shows the trend in solubility for sunflower oil in SC-CO_2 as a function of temperature and pressure. Again, an increasing yield of oil can be attained by conducting the extraction at higher temperatures. It is interesting to note that when the δ for SC-CO_2 is 8.7 $cal^{1/2}/cm^{3/2}$, it matches the solubility maxima recorded by Stahl et al. [18] for sunflower oil in SC-CO_2 (Fig. 4). This is very close to the theoretically predicted δ and reduced density conditions for solubilizing triglycerides as a solute class as given in Table 1.

It should not be inferred from the above discussion that a partial fractionation of mixtures of lipids is not feasible nor undesirable by selective SFE. This is usually accomplished by selecting discrete intervals of extraction pressure or density for a finite amount of time and collecting the resultant fraction. As shown in Figure 5, an enrichment of components in a triglyceride mixture is possible by changing the reduced pressure, P_r ($P_r = P_{exp}/P_c$), or reduced density and, ultimately, the solubility parameter of the fluid, over the range of associated values presented. Thus, a mixture enriched in the C_{34} triglyceride is initially achieved using a reduced fluid density of 1.87, whereas as the extraction is continued at higher values of reduced density, the higher-molecular-weight triglycerides are preferentially extracted. The resultant compositions of the extracts noted in Figure 5

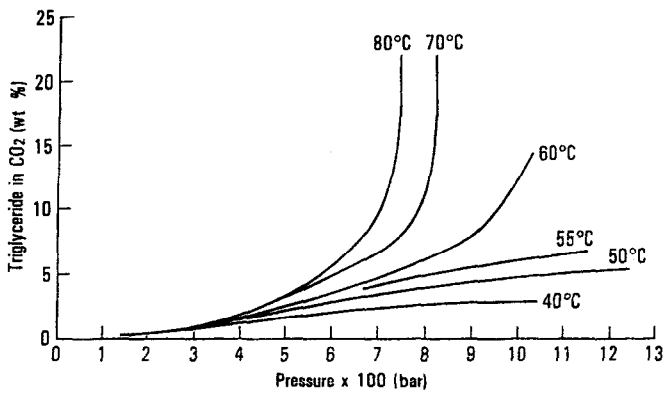

Figure 3. Solubility of soybean oil triglycerides in SC-CO_2 as a function of temperature and pressure.

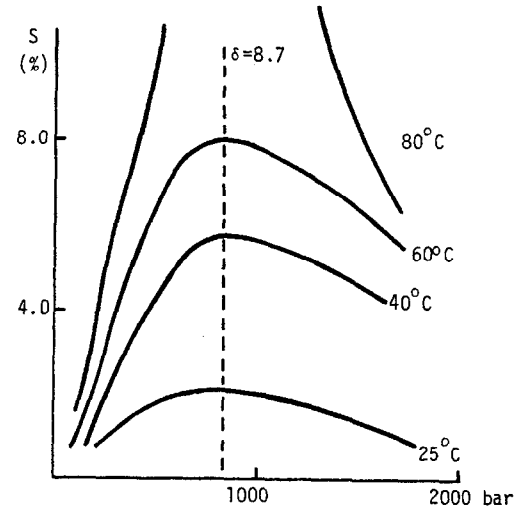

Figure 4. Solubility of sunflower oil (wt%) versus extraction pressure.

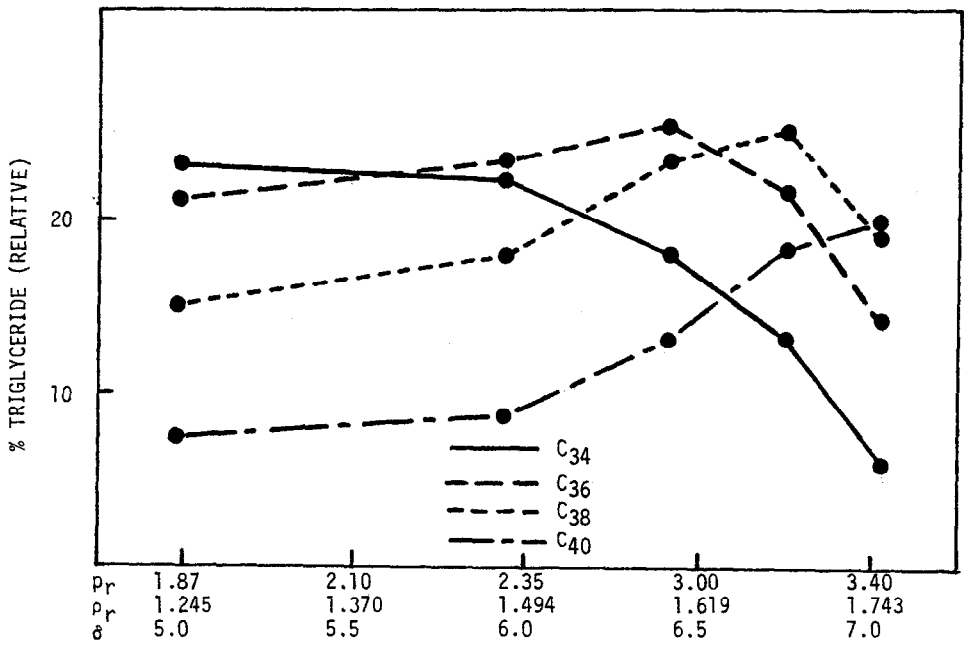

Figure 5. Percent triglyceride extracted versus extraction fluid reduced pressure, density, and solubility parameter.

are really a function of the chosen extraction density and the time over which the extraction is conducted.

Perhaps the most important result that can be ascribed to the mass transfer characteristics of supercritical fluids are the kinetics of extraction. This is illustrated in Figure 6 for the SFE of a high-value lipid product, evening primrose oil (EPO), from its crushed seed. These results are taken from the author's modeling study [19] of the extraction kinetics of EPO, but the results are generally applicable to the extraction of most lipid species, including pure compounds. The actual experimental data are designated by points representing different extraction pressures (1 MPa = 145 psi), and the lines are theoretical fits to the various extraction curves at 40°C based on the models of Brunner [20] and Hong et al. [21]. It can be seen in Figure 6 that the rate of EPO extraction (i.e., mass of EPO extracted versus time) is a function of the extraction pressure, suggesting that the initial predominating factor governing extraction rate is the oil's solubility in the SC-CO_2. Indeed, as shown in Figure 3, higher extraction pressures correlate with higher levels of triglyceride solubility in SC-CO_2; curves such as those in Figure 6 can be used to determine lipid solubilities in supercritical fluids [21]. Figure 6 also reveals that later in the SFE rate curve, as the oil becomes depleted from the seed matrix, there is the onset of a mass transfer region where further extraction of the residual oil is difficult. This can be due to several factors, including, particularly, the shape and particle size of the comminuted seeds; the problem has been elegantly described by Reverchon et al. [22]. This retardation of the oil-extraction kinetics has been termed the "diffusion phase" by Stahl et al. [23], or the nonequilibrium region of the extraction curve by the author.

As noted previously, SC-CO_2 reigns supreme as the principle processing agent in critical fluid technology. However, like any other extraction solvent, SC-CO_2 cannot be utilized for all tasks and is a relatively poor solvent for polar compounds. For certain applications, the addition of a minimal amount of a polar cosolvent (usually an organic solvent with a critical temperature, T_c, higher than CO_2) suffices to improve the extraction

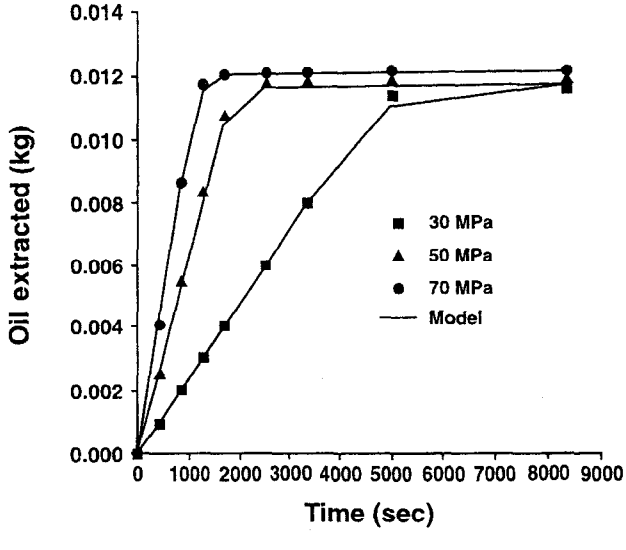

Figure 6. Extraction rate curves for evening primrose oil at 50°C as a function of extraction pressure; experimental data versus predicted extraction curve.

of compounds from natural products. The number of GRAS (generally regarded as safe) cosolvents suitable for this purpose is rather limited (e.g., water, ethanol, acetic acid). Cosolvents can be used in conjunction with SC-CO_2 to achieve fractionation of lipids, either in a single-step or multistep extraction process to produce the desired end result, a most notable example being the deoiling of seed moieties followed by SC-CO_2/ethanol solubilization of polar phospholipids, which have residual solubility in neat SC-CO_2 [15].

Propane has also been evaluated for critical processing of lipids, as it has a lower P_c (P_c = 48 atm, T_c = 97°C) than CO_2 and the solubility of lipids in near-critical propane is higher than in CO_2. Propane has been used for the extraction and fractionation of fish oil in the Selexol process [24], and a CO_2/propane mixture has been used for the deoiling of lecithin and fractionation of glycerides [25]. However, despite these advantages and applications, CO_2 seems to be more acceptable to the food industry than propane because it is nonflammable and a "green" solvent.

Other alternatives, which embrace the "green" processing concept, are binary fluid mixtures and specific fluorocarbon fluids. The use of liquefied gases (e.g., LCO_2) and pressurized liquids (i.e., liquids under an appropriate external pressure, so as to not vaporize at extraction temperatures above their boiling point) can produce superior results in specific cases, compared to conventional liquid solvent extraction [26]. For the processing of thermally sensitive lipids, the selection of extraction temperature is critical to avoid degradation of the solutes. It is for this reason that liquefied gases, such as LCO_2 are used at near-ambient or subambient temperatures in natural-product extractions [27–29]. In addition, pumping a liquefied gas does not require as much expenditure of energy as pumping and compressing a gas for specific processing conditions [30]. Recently, water has been used under subcritical conditions for the extraction and reaction of lipid moieties; however, these higher temperature conditions can alter the composition of some lipid material [31] if due caution is not exercised.

3 CRITICAL FLUID EXTRACTION OF LIPIDS AND OILS

The critical fluid extraction of lipids and oils has been reviewed by the author [32] and is the subject of an extensive monograph edited by King and List [33]. The reader is referred to these sources and the recent tome by Mukhopadhyay [13] for a historical appreciation of the SFE of lipid-bearing substrates. With respect to biotechnology applications, it is often the information presented in the proceeding section and the use of SFE as the fundamental basis for conducting fractionations and reactions in supercritical fluids that is of critical importance. For this reason, it is worth commenting on the equipment and mechanical aspects of SFE.

Evaluation of the potential and efficacy of SFE is often done on a bench scale, laboratory apparatus employing a tubular extraction vessel, and a continuous flow of the extraction fluid. In Figure 7, a schematic of a simple, bench-top extraction system that has proven very versatile in our laboratories is presented. The fluid source (e.g., CO_2), A, can be either in the gaseous or liquefied state, the liquid state being more appropriate when using larger extraction systems. A compressor or liquid pump, C, delivers the fluid through a tandem switching valve, SV-1 and SV-2, into the extraction cell held at a controlled temperature. The fluid passes through the cell containing the material to be extracted and is passed on through a similar switching valve arrangement to either a micrometering valve, MV, or back-pressure regulator, where the pressure of the fluid is reduced. These back-pressure regulators or metering valves are usually heated to compensate for

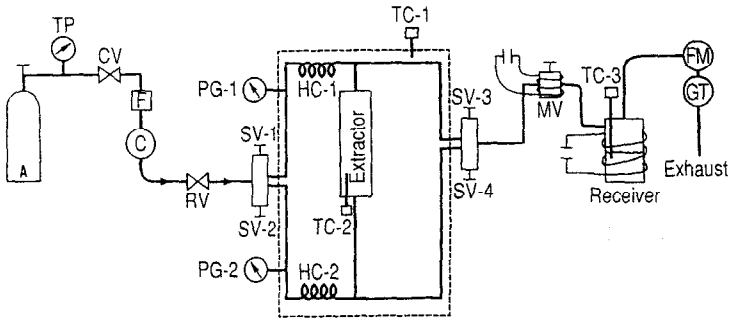

Figure 7. Laboratory bench-top supercritical fluid extraction system.

the attendant Joule–Thomson cooling effect that occurs when depressurizing the fluid. The fluid is then directed to a collection (receiver) vessel, which can exist in several formats, and may be packed with internals or kept at a very low temperature (−15°C) to eliminate entertainment of the extracted components in the rapidly expanding critical fluid. Gas flow under ambient conditions is assessed with the aid of a flow meter, FM, and fluid totalizing module, GT. This type of unit can be reformatted for different types of experiment involving critical fluids and has proven to be extremely flexible for a modest cost [34].

Although the above-described system is entirely adequate for laboratory SFE experiments, it does not meet the requirements of an actual production SFE operation. In these cases, the fluid is reused by circulating it over and over again through the substrate in the extraction vessel. Thus, the solvent (fluid) supply remains constant except for small losses, an attractive feature of process SFE. To illustrate this principle in greater detail, a schematic of the semicontinuous pilot plant at our research center is illustrated in Figure 8. Here, continuous SFE is assured by the use of several 4-L extraction vessels utilized in tandem. The extraction solvent can be directed in a sequential fashion to one or more of the extraction vessels; thus, vessel A can be extracted while another vessel, B, is being loaded with the material to be processed. The third pictured vessel, C, will occupy an intermediate processing state undergoing either pressurization or depressurization. The receiver vessel, R, must be of sufficient size (2 L) to allow separation of the extracted solutes by depressurizing the fluid. A sorbent-filled column is inserted on the low-pressure side of the fluids recycle line to remove unwanted odoriferous volatile compounds. Note that a provision is made for making up fluid lost during the removal of the extract from the receiver vessel. Utilizing the above scheme and equipment has permitted the exhaustive deoiling of oil seeds such as soya in as little as 10 min, using mass flow rates of SC-CO_2 of 0.5 kg/min at 80°C and 10,000 psi [35]. Actual industrial-scale SFE installations use lock hoppers or screw conveyors under reduced pressure to continuously feed solid substrates into multiple extraction vessels.

The SF processing of specialty oils is perhaps of most interest when applying SFE in the field of biotechnology. This is because these oils are marketed as nutraceuticals and sold on the basis of their unique fatty acid composition and/or presence of lipid-soluble minor components in their composition, as these ingredients have implied health benefits. Such specialty oils also find applications in medicine and skin care products and they feature antioxidant activities that are reported to be inhibiting against cancers. Re-

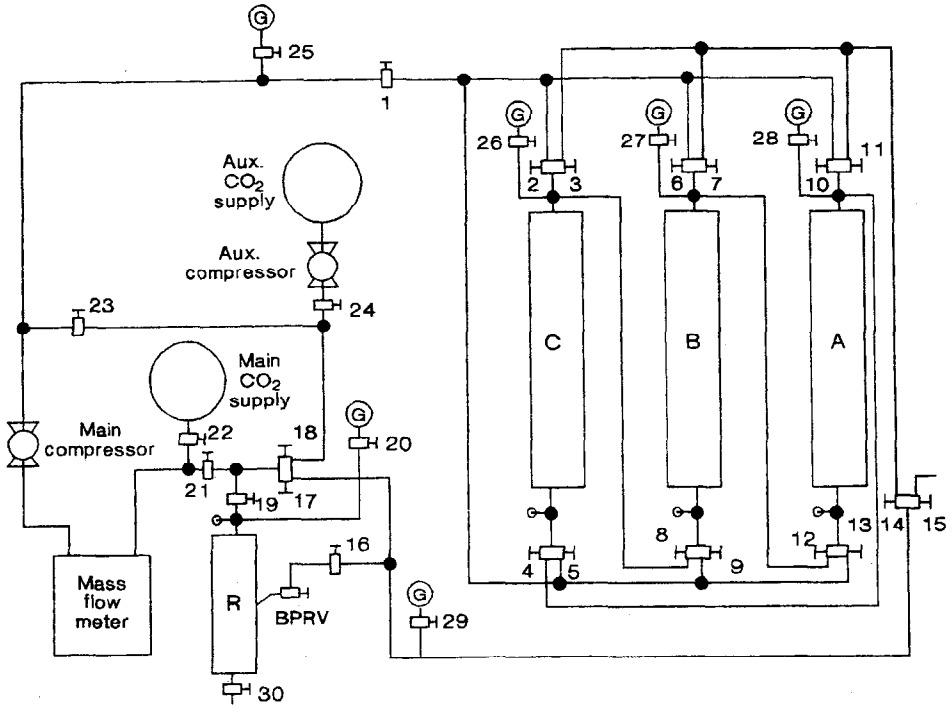

Figure 8. Batch semicontinuous supercritical fluid pilot plant with provision for extraction fluid recirculation.

cently, the presence of minor lipid components in some speciality oils has also been shown to reduce serum cholesterol levels in human subjects [36].

Examples of these therapeutic oils are those derived from oat and barley oils, because they are rich in tocopherols and tocotrienols. Oat oil has been extracted using SC-CO_2 by Fors and Eriksson [37], whereas barley oil can be extracted from whole or pearling flour because the majority of the extractable lipids are located in the outer layers of the seed kernel. Another source of natural antioxidants is rice bran oil which has been extracted using SC-CO_2 to yield an extract rich in tocopherols, oryzanol, and sterols (campesterol, stigmasterol, β-sitosterol) [38,39]. Recently, Xu and Godber [40] have extracted rice bran with both SC-CO_2 and organic solvents and have shown that the γ-oryzanol content of the SC-CO_2-derived extract is four times greater than the extract obtained with organic solvents. In a similar fashion, neat SC-CO_2 was shown to yield a ferulate–phytosterol ester containing extract from corn fiber by Moreau et al. [41] or a sterol content in a SC-CO_2 extract of saw palmetto, which was approximately four times that of a corresponding ethanol extract. Such SC-CO_2-derived oil extracts offer a potential alternative to the cholesterol-reducing properties of chemically altered tall oil extracts that have been recently commercialized and sold in margarine formulations [42].

As previously noted, EPO can readily be extracted with SC-CO_2. The interest in this oil is due to its high γ-linolenic acid content. Extracts obtained between 200 and 690 atm and 40–60°C have been characterized by Favati et al. [43]. Similarly, other oils that are rich in γ-linolenic acid (borage, blackcurrent, and flax) have been extracted using

critical fluids. In our laboratory, oils have been obtained using SC-CO_2 extraction from wheat germ, avocado, and sorghum bran that have implied therapeutic value. It also has been reported that critical fluids can also be used to extract oils which are devoid of cholesterol [44] from fungi or marine-derived plant material, such as spirulina. Extraction of natural lipid-type pigments, such as carotenoids and xanthophylls from similar type substrates, has been reported as well as from freeze-dried substrates of alfalfa leaf protein concentrate [45]. An excellent review of the SFE of natural pigments is provided by Mukhopadhyay [13].

4 FRACTIONATION OF LIPIDS USING SUPERCRITICAL FLUIDS

Prior to the mid-1980s, critical fluid processing was largely accomplished using SFE. As noted previously, selective fractionation of extracts was achieved by either altering the extraction fluid density as a function of processing time or, in some cases, by selectively decreasing the pressure after the extraction stage to achieve the desired extract. The latter fractionation technique makes use of successive receiver vessels in which the extracting fluid density is progressively lowered by manipulating the temperature and pressure. Examples of this technique include the dewaxing of essential oils by Della Porta et al. [46] and the isolation of tocopherols from olive oil by Ibanez and co-workers [47]. Useful separations have been attained using the above techniques, but largely between compounds which differ significantly in their physicochemical properties (e.g., molecular weight, vapor pressure, or polarity).

Fractionation processes utilizing critical fluid media have been improved by combining principles utilized in supercritical fluid extraction with other separation techniques. These improved methods often make use of fractionating columns or chromatography to yield improvements in the resolution of the desired molecular species. The fractionating column or tower approach is somewhat analogous to operating a distillation column, but there are differences when using critical fluid media. For an understanding of the fundamentals involved in using this technique, one should consult the primer by Clifford [48].

Figure 9 illustrates the components and principles involved in column-based fractionation using the simple column in our research laboratory. Although this is not the most sophisticated SFF column approach, it will serve as an example of how the process works. In this case, SC-CO_2 is brought to the desired extraction pressure where upon fluid is directed to flow upward inside the fractionating column. The fractionating column contains a packing to facilitate contact between the SC-CO_2 and the components being separated. The components to be separated are injected with a pump into the flowing SC-CO_2, prior to its entry into the column (components 5 and 10 in Fig. 9). The fluid–solute mixture then enters the first heated zone of the fractionating column and the separation process is initiated. The SC-CO_2–solute mixture then ascends the column encountering zones of increasing temperature, which amplify the separation of solutes based on their relative solubilities in SC-CO_2 and respective vapor pressures. In effect, the column is operating as a density gradient in SC-CO_2 because the fluid is kept isobaric.

The fractionating column described in Figure 9 can be operated in either the batch or semicontinuous mode with cocurrent flow of the solute and supercritical fluid streams. Using this approach, we have demonstrated the enrichment of lipid monoglycerides (MAG) from a mixture of glycerides [49]. The effect is nicely demonstrated by capillary SFC profiles shown in Figure 10. Here, the feed material is a mixture of glycerides in which the MAG content is 45 wt% of the mixture. As the fractionation of the mixed

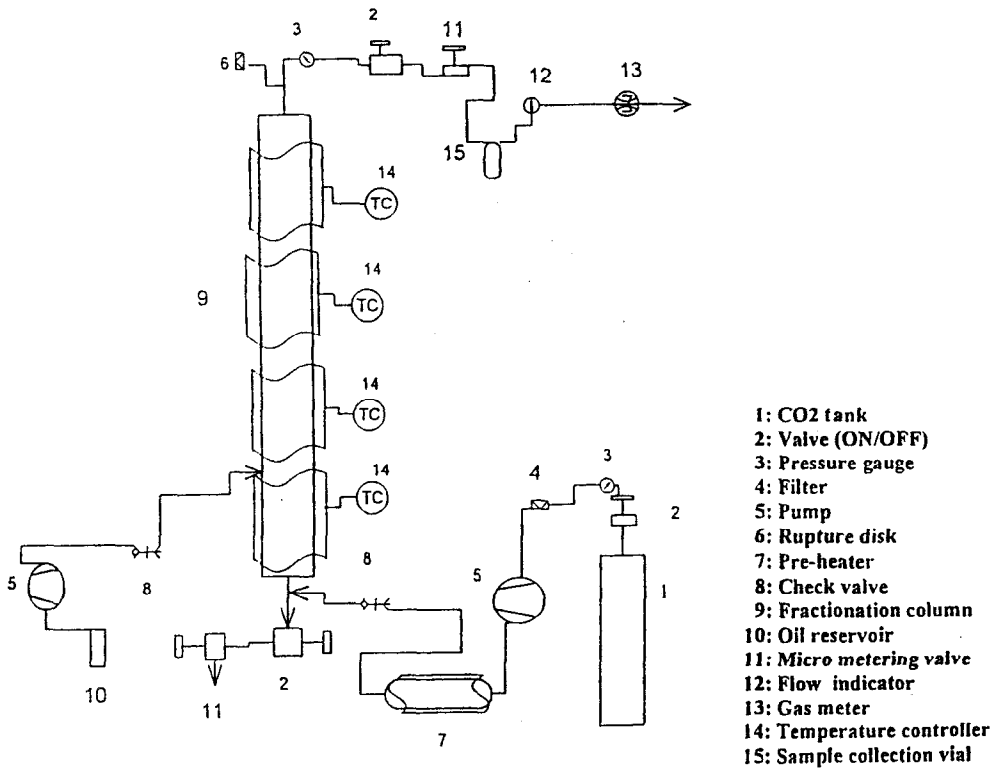

Figure 9. Schematic of a cocurrent critical-fluid-packed fractionation column system.

glyceride feed proceeds, the extract that is removed from the top of the column is considerably enriched in MAG content. Using this approach, an extract containing 95 wt% of MAG can be achieved [50].

A similar fractionation approach [51] has been recently used to produce enriched nutraceutical extracts from deodorizer distillate (DD). DD is a complex by-product from the refining of vegetable oils and contains many components, as illustrated by the capillary SFC chromatogram in Figure 11. Many of these components are desired in a more pure or enriched fraction. Therefore, in a recent study, we used a fractionating column to remove undesired free fatty acids from DD while enriching the free sterols and steryl ester content of the raffinate (top product).

The results of this study are presented in Table 2 for both rice bran and soybean oil DD. In this case, the starting material (the feed into the column) contained between 32 and 38 wt% free fatty acids and between 13 and 18 wt% sterols, whereas the raffinate collected after the fractionation had a free fatty acid content of between 5 and 8 wt% and sterol–steryl ester enrichments of between 27 and 34 wt%. These results could undoubtably be improved upon by employing an even longer fractionating column or by preferentially operating a column in the countercurrent mode, which other investigators have employed in Germany and Italy. For example, tocopherols have been enriched from soybean oil DD by Brunner and colleagues [52] and the same research group separated the ethyl esters of fish oil basestock using the countercurrent column approach [53].

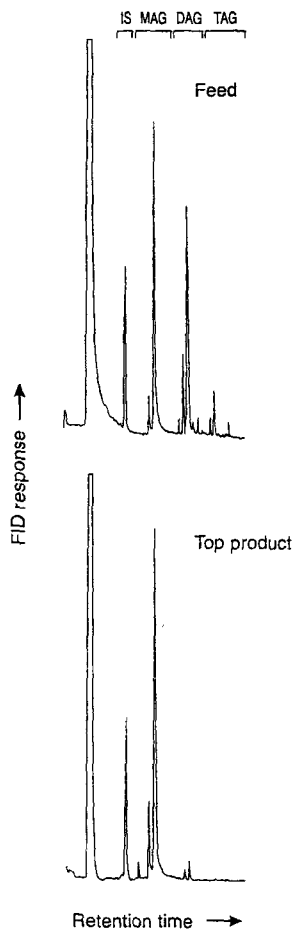

Figure 10. Capillary supercritical fluid chromatographic profile of feed and top (extracted) product glyceride profiles using critical-fluid-packed column fractionation. MAG = monoacylglycerides, DAG = diacylglycerides, TAG = triacylgylycerides, IS = internal standard.

Another SFF option is to employ chromatography in the preparative or production mode, in its own right or coupled with a preliminary SFE enrichment stage. Our research group at NCAUR has utilized the latter approach several times to achieve extracts with target compounds enriched at levels that were not possible by using SFE alone. There are a number of different SF chromatographic-based methods that could be used for this purpose, but the process described in Figure 12 has been designed with scale-up and economics in mind. As shown in Figure 12, flaked soybeans are initially extracted at a relatively low pressure to enrich the target components of interest, tocopherols. This fraction is then moved sequentially on to a sorbent-filled column for further fractionation to yield a tocopherol-enriched extract. Enrichment factors relative to the tocopherol content in the original soybean flakes are tabulated in Table 3. Note that these are only modest for application of the single SFE-based separation; however, significant enrichment of the desired components is attained by applying the chromatographic fractionation step [54].

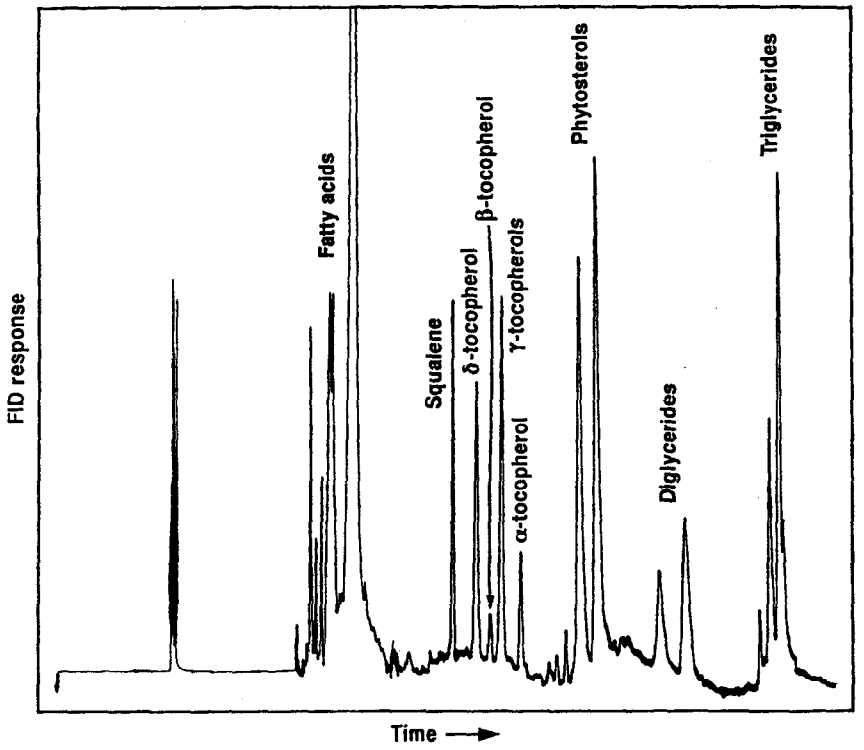

Figure 11. Capillary SFC profile of soybean oil DD.

Using this approach, we have been able to enrich other lipid moieties in addition to tocopherols, such as phospholipids or steryl esters from vegetable oils, seeds, and by-products of the milling or vegetable oil refining processes. Figure 13 illustrates the case for the separation, enrichment, and fractionation of phospholipids (PLs) from vegetable oils or seeds [55]. Here, soybean flakes are initially extracted with SC-CO_2 to remove the oil, followed by extraction of the PLs from the deoiled flakes with a SC-CO_2–ethanol mixture. This second extraction produces an extract which is enriched in PLs, because PLs are not appreciably soluble in neat SC-CO_2 but can be solubilized in SC-CO_2 ethanol mixtures.

Table 2 Raffinate Compositions (wt%) from Fractionation Tower Separation of Vegetable Oil Deodorizer Distillates (DD) Using SC-CO_2

Component	Rice bran DD	Soybean DD
Free fatty acids	5	8
Sterols	20	31
Steryl Esters	7	3
Triglycerides	38	30

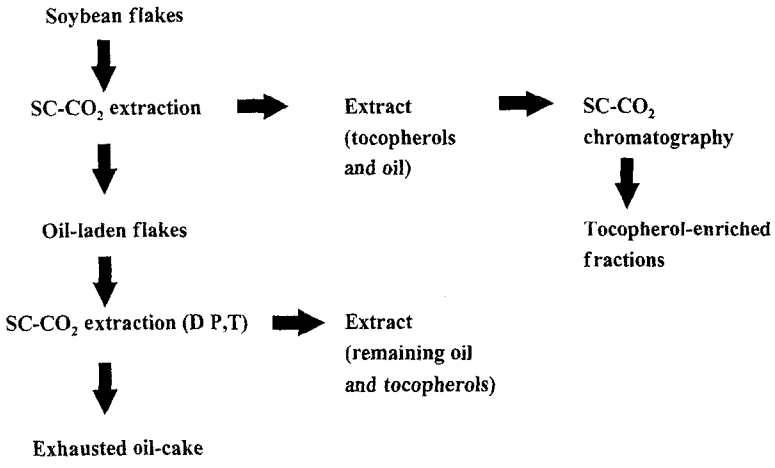

Figure 12. Tocopherol enrichment/fractionation scheme using supercritical fluid techniques.

As shown in Table 4, the second SFE using SC-CO_2–ethanol produces an extract containing a total 43.7% by weight of PLs. This is a considerable enrichment relative to the concentration of the PLs in the starting oil or seed matrix. Further PL enrichment is facilitated as noted earlier, by transferring this extract enriched in PLs to an alumina preparative SFC column, where SC-CO_2 modified with 5–30 vol%, 9:1 ethanol:water eluent is used to elute and fractionate the PLs. In the case of the SFC enrichment step, eluent fractions can be collected as a function of time and their PL content quantitated. As indicated by the data given in Table 4, collection of discrete fractions during the SFC process can produce purities in excess of 75% for the individual PLs, phosphatidylcholine and phosphatidylethanolamine. It should be noted in the described process that in the SFC steps, only GRAS (generally regarded as safe) solvents are being used for the enrichment process.

Recently, a similar SFE/SFC process has been used to isolate sterols and phytosterol esters from corn bran and fiber oil [56]. For example, by using both SFE and SFC, it has been possible to isolate a fraction containing up to 53% by weight of ferulate phytosterol esters (FPE) from corn fiber oil. Moreau et al. have isolated similar moieties in a total oil extract using SC-CO_2, which is called "Amaizing Oil" [57]. Intended for use as a cholesterol-lowering agent, this extract unfortunately contains mostly triglycerides and

Table 3 Enrichment Factors of Tocopherols from Soybeans by SFE and SFE/SFC

Tocopherol	SFE	SFE/SFC
Alpha	4.33	12.1
Beta	1.83	2.4
Gamma	3.94	15.0
Delta	3.75	30.8

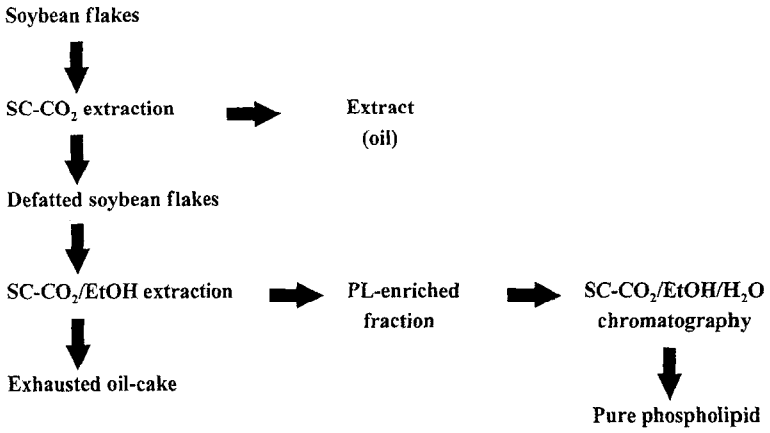

Figure 13. Phospholipid enrichment/fractionation scheme using supercritical fluid techniques.

about 6 wt% FPEs. Whereas this extract may have some utility in the nutraceutical marketplace, it is limited for formulating or chemical testing purposes due to the limited enrichment of FPEs. The tandem SFE/SFC process provides an even greater enrichment of the active principle and could be improved on further by using successive chromatographic purification.

Recently, the principle of simulated moving-bed (SMB) chromatography has been applied to the purification of lipids such as fish oil esters. A special facility that deserves some mention is the relatively new KD-Pharma/Industries Quimicas Asociadas plant for producing concentrates of ω-3 fatty acid esters [eicosapentaenoic (EPA) and docosahexaenoic acids (DHA)] from fish oil in Tarragona, Spain. This production facility employs a combination of SFE/SFC to produce fish oil ester mixtures of 95% purity. The process uses a proprietary silica-based packing material in the SFC stage to separate the ω-3 fatty acids from the ω-6 moieties, as described in detail by Lembke [58] for the pilot-plant-scale operation. Increasing the EPA content of this natural product improves its nutraceutical functionality and is an excellent example of what can be achieved using supercritical-fluid-based fractionation methods.

Table 4 Relative Amount of Phospholipids from Soybeans in SFE Isolates and in SFC Collected Fractions

Phospholipid	SFE[a]	SFC
Phosphatidylethanolamine	16.1	74.9
Phosphatidylinositol	9.2	20.8
Phosphatidic acid	2.8	55.8
Phosphatidylcholine	15.6	76.8

[a] All data in percent of that component relative to other eluting constitutents (oil and unidentified peaks).

5 LIPID REACTION CHEMISTRY IN SUPERCRITICAL FLUIDS

The ability to conduct reactions in critical fluid media has been under extensive study over the past 9 years [59]. Three types of reaction (enzymatic, hydrogenation, and conversions) in subcritical water are of particular interest with respect to their application for lipid materials. Critical fluids offer some unique advantages when conducting reactions, including improvements in mass transfer of reactants and products and potential control of the final product distribution by altering the fluid density in which the reaction takes place. Additional options include the possibility of performing conversions at low temperatures, *in situ* regeneration of catalysts, and combining the reaction step sequentially with SFE or SFF.

It should be noted that the Gibbs free energy of reaction is also sensitive to pressure, as defined by Eq. (2):

$$\left(\frac{\partial RT \ln K_x}{\partial P}\right)_T = \nabla V \tag{2}$$

where K_x is the mole fraction equilibrium constant and ∇V is the excess partial molar volumes of the products over the reactants in the equilibrium mixture. Therefore, regardless of any benefits that come from conducting a reaction in critical fluid media, the application of pressure will have an influence on the reaction.

Experimental parameters which can influence the conversion of lipid-based substrates in critical fluid media are listed in Table 5. The composition of fluid phase is important because it ultimately influences the solute (reactant) solubility and introduction of reagents (H_2) into the critical fluid system. Therefore, phase equilibria and solute solubility relationships are important, not only with respect to assuring that adequate solute (reactant) solubility occurs in the critical fluid media but also that an adequate throughput of converted product is feasible to make the synthetic process kinetically feasible and economical. Other important relationships are the optimization of reaction conditions via proper selection and activation of the catalyst (if required) and the moisture content of the substrate (in the case of certain types of enzymatic catalysis). The flow rate in tubular reactor systems is also critical, not only with respect to the critical fluid but also for the introduction of reactants and their solubilization in the critical fluid media. In addition, the flow rate is linked to product throughput and must be optimized to allow proper kinetic conversion of the reactants.

One promising area for applying supercritical fluid reactions (SFR) for the conversion of lipids is the use of enzymes for initiating reactions such as esterifications, transesterifications, oxidation, alcoholysis, and hydrolysis. Immobilized enzymes on porous supports for conducting conversions in a tubular flow reactor are particularly amenable for

Table 5 Experimental Parameters That Influence Lipid Reaction Chemistry in Critical Fluids

Fluid type and composition	Optimization of reaction conditions
Pressure	Catalyst type and activity
Temperature	Moisture content of substrate
Phase equilibria	Effect of flow rate
Solute (lipid) solubility	Solute throughput

conversions using critical fluid media. The coupling of enzyme catalysis with SC-CO_2 is particularly attractive because both are "natural" agents that avoid the use of chemical solvents or catalyst residues in the final product. The above transformations can be conducted in the presence of a lipase, but particular attention must be paid to the temperature, pressure, and presence of water in the supercritical fluid system. Of particular note is Novozym SP 435, a lipase derived from *C. antarctica*, as a catalyst supported on polyacrylate resin, an enzyme that has proven to be a particularly effective catalyst in the presence of SC-CO_2 [59–62].

Jackson and King [60] have demonstrated the compatibility of an enzymatically catalyzed transesterification in SC-CO_2 on triacylglycerol (TAG)-based oils extracted directly from seeds (soybean) using an enzyme flow reactor system. Such transesterifications and simple esterifications can be conducted using Novozym SP 435 at pressures from 2500 to 5000 psi and temperatures from 40°C to 70°C. The utilization of higher temperatures and pressures has been reported [61] and is desirable in terms of increasing the solubility of the substrate (TAGs), but this can also reduce the service lifetime of the enzyme. An example of the conversion possible in a SC-CO_2/Novozym SP-435-based transesterification system is shown in Figure 14, where analytical capillary SFC shows a 97% conversion of soybean trigylcerides to the corresponding fatty acid methyl esters (FAMES) at 2500 psi and 60°C. This formation of fatty acid methyl esters is so complete, reproducible, and quantitative that it has served as a basis for analytical SFE/SFR methods developed in our laboratory for nutritional fat analysis [63].

Lipolysis has been used to successfully methylate other lipid moieties such as sterols and phospholipids [64]. Such results pave the way for esterification of these compounds to other synthetic compounds having different fatty alcohol chain lengths. Recently, steryl esters have been synthesized by our research group using SC-CO_2 and various lipases,

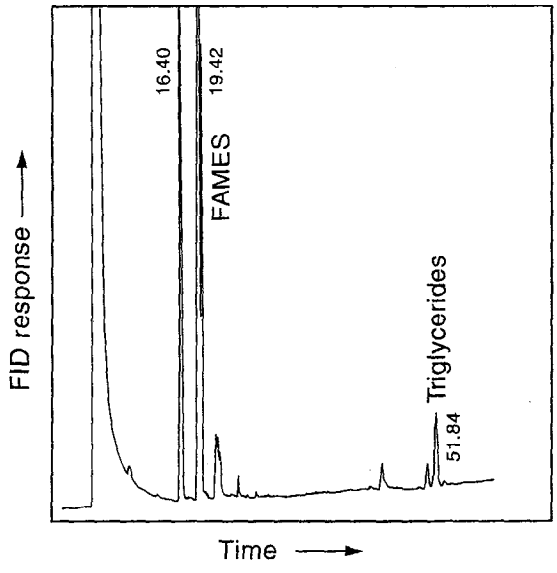

Figure 14. Capillary SFC profile of end-product mixture from enzymatic conversion of TAGs to FAMES.

including partially dehydrated soapstock feeds containing fatty acids which have also been esterified using the above conditions [65].

The presence of moisture in natural product substrates can have an effect on enzymatic-based synthesis. A previous study [66] showed that there is a minimal amount of water that must be associated with the enzyme in the presence of the critical fluid to assure retention of activity. However, excessive water can denature the enzyme, leading to loss of activity and conversion of reactants. This is illustrated by the results in Table 6, in which the effect of added water on the methanolysis of corn oil to form FAMES is described. Note that in terms of volume percent of water in SC-CO_2, this is quite a small quantity (0.05 vol%) and must be rigorously controlled to prevent loss of activity and conversion. Fortuitously, the solubility of water in SC-CO_2 is quite small [67] and this aids in maintaining the activity of the enzyme for long periods of time when extracting and reacting lipid moieties from natural products. Another convenient way of maintaining the hydration level critical for maintenance of the enzyme's activity in the presence of a critical fluid is to add the requisite amount of water via a syringe pump directly into the critical fluid.

The rate addition of reactants to a flow reaction system operating under critical fluid conditions can be quite critical in assuring the maximum yield of end products. For example, Jackson and King [60] have shown that the addition of methanol for conducting a transesterification of a vegetable oil must be optimized or the relative activity of the enzyme will not be realized. This is required to assure that there is an adequate stoichiometry of the reactants as well as time for these moieties to react during their passage over the supported enzyme catalyst. This is also true in the case of hydrogenation of triglyceride-based vegetable oils in SC-CO_2 or SC-C_3H_8 in the presence of a supported catalyst, as will be discussed shortly.

Another type of reaction that can be catalyzed by an enzyme in the presence of a supercritical fluid is the interesterification of vegetable oils to produce a "randomized" product having quite different physical and chemical properties than the starting materials. Jackson et al. [62] interesterified a variety of starting materials by dissolving them in SC-CO_2 and transporting them over immobilized beds of Novozym SP-435 lipase at 27.5 MPa (3988 psi) and 65°C. The end effect of this interesterification is quite striking because liquid vegetable oil feedstocks can be randomized to products of a semisolid nature having potential as margarine base stocks.

Table 6 Effect of Added Water on the Methanolysis of Corn Oil in SC-CO_2

Volume% water in carbon dioxide	Relative activity
0	100
0.05	99
0.10	81
0.20	56
0.30	18

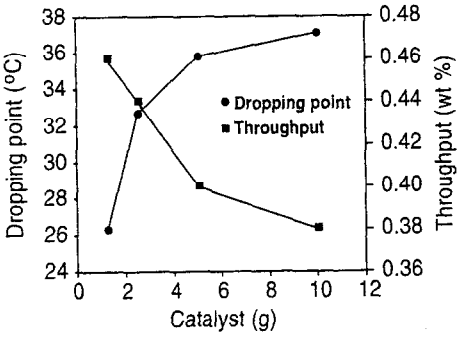

Figure 15. Effect of catalyst (enzyme) concentration on fat dropping point and end product throughput for randomization of palm olein in SC-CO_2 via enzymatic catalysis.

For palm olein, the resultant physical end products of the interesterified product is highly dependent on the amount of catalyst (enzyme) that is used. In this study [62], palm olein was randomized using varying amounts of Novozym SP-435 under the above conditions with an expanded CO_2 flow rate of 12.5 L/min. As would be expected, increasing the amount of catalyst resulted in a fat with a higher dropping point (a measure of solid–liquid content of the resultant fat) as indicated by the trend shown in Figure 15. The dropping point of the initial palm olein was 21.7°C, indicating that even the smallest amount of enzyme had a sizable effect on the dropping point of the triglyceride mixture. Also indicated in Figure 15 is that the production rate (throughput) of randomized palm olein through the SF reactor decreased with an increase in the quantity of enzyme used. Thus, it would appear that the dropping point and throughput are inversely related and that at 2.5 g of enzyme, the maximum throughput and dropping point can be realized for this particular interesterification.

Figure 16 shows the solid fat content of a high-stearate soybean oil and palm olein both before and after randomization. After randomization, both of these oils show an increase in solid-fat content, as measured by wide-line nuclear magnetic resonance, at

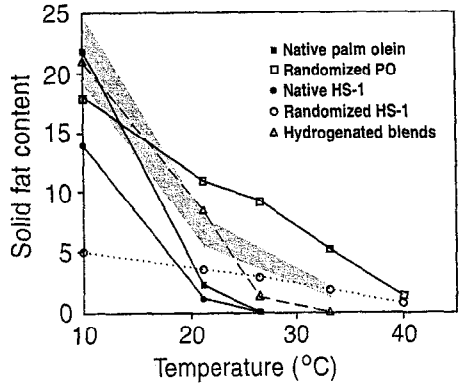

Figure 16. Solid-fat content of palm olein and HS-1 soybean oil before and after randomization in SC-CO_2 at 27.5 MPa and 65°C.

higher temperatures and a decrease at lower temperatures. The randomized HS-1 and palm olein (PO) begin to have a higher fat content than their native state at approximately 15°C and 20°C, respectively. Typical solid-fat content for soft-tub margarine oils obtained by blending hydrogenated and liquid soybean oils shows a thermal behavior indicated by the shaded area in Figure 16. At 26°C, both enzymatically randomized oils have higher solid-fat contents than the hydrogenated blend. It is apparent that the randomized palm olein product has a SCI that exceeds that typically found for commercial products and that the randomized HS-1 product approaches the SCI values found for soft commercial margarines.

Another way of producing semisolid oleochemical formulations for food use is through hydrogenation of native oils and fats. Harrod et al. [68] and Tacke and co-workers [69,70], have hydrogenated a variety of oleochemicals and shown that hydrogenation of fats/oils is feasible under supercritical conditions using SC-CO_2 and SC-C_3H_8. Recently, we have studied the hydrogenation of FAMES using binary fluid mixtures of SC-CO_2/H_2 and SC-C_3H_8/H_2 in a flow reactor under SF conditions at quite high temperatures (150–250°C) [71]. Using conventional inorganic catalysts, FAMES were exhaustively hydrogenated to yield the corresponding fatty alcohols using a critical fluid phase which can contain up to 25 mol% H_2. High conversion rates (>98%) were achieved quite rapidly due to the enhanced contact made between the fixed–bed catalyst and the methyl esters dissolved in the fluid mixtures. Greater product throughput was achieved by using the SC-C_3H_8/H_2 mixture due to the higher solubility of FAMES in SC-C_3H_8 versus SC-CO_2; however, the SC-CO_2/H_2-based process gives less by-product *n*-alkanes relative to the SC-C_3H_8/H_2 system.

The above hydrogenation reaction can be combined advantageously with an initial transesterification step to synthesize FAMES directly from vegetable oils extracted in SC-CO_2. In this case, soybean oil triglycerides were methylated using the conditions established by Jackson and King [60] and then transported from the supported enzyme reactor to flow through the hydrogenation reactor. Hydrogenation of the FAMES was then accomplished by continuously facilitating the total reduction of the FAMES to the saturated alcohols (hexadecanol and octadecanol), splitting off methanol. Using this procedure, the methanol by-product can be fed back into the first stage of the synthesis process (transesterification) to produce FAMES.

In the studies mentioned so far, SC-CO_2 has been the predominant critical fluid media used due to its low environmental impact. Another medium that meets this criteria is subcritical water, which can be defined as hot compressed water held between 1 and 218 atm and between its normal boiling point and critical temperature of 374°C. Under these conditions, water does not boil away and can act as a solvent whose solubility characteristics are determined predominantly by the extraction or reaction temperature of the water [72]. Research conducted in our laboratory has utilized subcritical water for the hydrolysis of vegetable oils to synthesize fatty acid mixtures [73]. The results of this subcritical water hydrolysis are given in Table 7 and demonstrate how complete this conversion can be under a variety of conditions. Note that residence times under 10 min can produce over a 90% conversion of the vegetable oil feedstock (in this case soybean oil) to the component fatty acids. Although this approach uses higher water-to-oil feed ratios than those currently used in industrial hydrolysis processes, it requires no catalyst.

In conclusion, the future would appear bright for reaction chemistry in critical fluid media. As noted earlier, there are a variety of media that can support synthesis above and below their respective critical points (SC-CO_2, SC-C_3H_8, and subcritical H_2O), with the

Table 7 Conversion of Soybean Oil to Free Fatty Acids Using Subcritical Water in an Open-Tubular Flow Reactor

Residence time (min)	12.6	9.9	7.5
Temperature (°C)	335	335	335
Pressure (atm)	125	125	134
Water:oil ratio	2.5:1	5:1	2.5:1
% Free fatty acid	98	100	90.4

resultant synthetic processes being potentially capable of being coupled with SFE and SFF in pre-reaction and postreaction enrichment schemes to yield a variety of lipid-processing possibilities.

6 CONCLUDING REMARKS

In the preceding sections, it was demonstrated how critical fluids, applied as an overall technological approach, can be extremely useful in isolating, fractionating, and converting lipids into useful industrial products. The high capital costs of implementing critical fluid technology makes it imperative that plants and processing facilities be adaptable to other roles besides just the extraction mode. The examples given in this chapter suggest that such options are feasible and can be coupled to considerable advantage.

The conditions for solubilizing lipids under supercritical conditions do not always favor the use of biocatalysis, primarily because high lipid solubility in SC-CO_2 is commensurate with the use of high pressures and temperatures, which may denature bioactive proteins. Recent developments in the field of high-pressure food processing [74] suggest that enzyme and protein functionality may be more resilient than previously thought and hold the key to the development of better thermophilic and hyperbaric enzymes. Compressed, subcritical water's use may also be extended in the reaction area in the near future.

Supercritical fluid technology can also serve the needs of lipid biotechnology through its use in analytical chemistry. As has been amply demonstrated in this chapter, analytical SFC is an excellent and rapid form of chromatography for separating various lipid classes without the need for extensive sample preparation. Additional examples of its use in lipids separation and analysis are contained in the review by King and Snyder [75]. Analytical SFE has had a major impact as one of several competing technologies to reduce the time, cost, labor, and extensive use of organic solvents in sample preparation methods [76]. Major application areas for analytical SFE have been in fat and lipid analysis [77], toxicant residue determination [78], and as a benign method to study lipid degradative processes [79].

In the author's view, analytical supercritical fluid technology can serve as a testing ground for assessing the feasibility of conducting extractions, fractionations, and reactions in critical fluid media. The use of analytical supercritical fluid instrumentation, when automated and miniaturized, can save considerable effort, particularly when working with expensive and scarce lipid biochemicals. Such equipment allows a combinatorial technological approach to evaluating the myriad of possibilities presented by lipid biotechnology while blending a third technology with the two previously mentioned.

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